Cyclic movement stimulates hyaluronan secretion into the synovial cavity of rabbit joints

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The novel hypothesis that the secretion of the joint lubricant hyaluronan (HA) is coupled to movement has implications for normal function and osteoarthritis, and was tested in the knee joints of anaesthetized rabbits. After washing out the endogenous synovial fluid HA (miscibility coefficient 0.4), secretion into the joint cavity was measured over 5 h in static joints and in passively cycled joints. The net static secretion rate (11.2 ± 0.7 μg h^{-1}, mean ± S.E.M., $n = 90$) correlated with the variable endogenous HA mass (mean 367 ± 8 μg), with a normalized value of 3.4 ± 0.2 μg h^{-1} (100 μg)$^{-1}$ (%$\dot{q}_{HA}$). Cyclic joint movement approximately doubled the net HA secretion rate to 22.6 ± 1.2 μg h^{-1} ($n = 77$) and raised the normalized percentage $\dot{q}_{HA}$ to 5.9 ± 0.3 μg h^{-1} (100 μg)$^{-1}$. Secretion was inhibited by 2-deoxyglucose and iodoacetate, confirming active secretion. The net accumulation rate underestimated true secretion rate due to some trans-synovial loss. HA turnover time (endogenous mass/secretion rate) was 17–30 h (static) to 8–15 h (moved) The results demonstrate for the first time that the active secretion of HA is coupled to joint usage. Movement–secretion coupling may protect joints against the damaging effects of repetitive joint use, replace HA lost during periods of immobility (overnight), and contribute to the clinical benefit of exercise therapy in moderate osteoarthritis.

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Hyaluronan (HA), a hetero-disperse polysaccharide of $> 10^6$ Da, is composed of repeating D-*N*-acetylglucosamine D-glucuronic acid disaccharides. HA has a central role in synovial joint function. Its secretion drives cavity formation during embryogenesis (Pitsillides, 2003); it contributes to the high viscosity and lubricating properties of synovial fluid; it organizes the synovial interstitial matrix and profoundly reduces its hydraulic permeability (Coleman, 2005); and it retains synovial fluid during joint flexion through a concentration polarization process (Coleman *et al.* 1999). HA also has key roles in cartilage biomechanics, cardiac embryogenesis, cell motility/migration in wound healing, angiogenesis and cancer (Tammi *et al.* 2002). It is present at a high concentration in the synovial fluid (~3.6 mg ml^{-1}, rabbit knee) but at a much lower concentration in the synovial interstitial matrix (0.27 mg g^{-1}) and articular cartilage (0.42 mg g^{-1}) (Price *et al.* 1996*a*). The fluid's HA concentration and viscosity decline substantially, however, in arthritis, reducing its lubricating capacity (Dahl *et al.* 1985) and facilitating nociceptor activity (Pawlak *et al.* 2002). Consequently the intra-articular injection of HA is currently a widespread therapy for moderate osteoarthritis (OA) (Day *et al.* 2004).

HA is selectively retained inside the joint cavity due to its enormous molecular volume, which causes it to be reflected (ultrafiltered) by the synovial membrane during fluid drainage (Sabaratnam *et al.* 2004). Since the synovial reflection coefficient is less than unity (~0.9), some HA also escapes slowly from the joint cavity (Brown *et al.* 1991; Sabaratnam *et al.* 2004). Fresh HA is synthesized and secreted continuously to replace the loss and maintain the intra-articular concentration, but little is known about how the synovial secretion rate is regulated *in vivo*.

HA is synthesized by fibroblast-related synoviocytes (called simply synoviocytes hereafter), which form 67% of the cell population lining the joint cavity (Levick & McDonald, 1989). Synoviocytes strongly express hyaluronan synthase (HAS), a ~63 kDa membrane-spanning protein, as well as enzymes supplying uridine diphospho(UDP)-sugar substrates (Pitsillides *et al.* 1993) and oxidative enzymes (Iwanaga *et al.* 2000). Of the three mammalian isoforms HAS1–3, rabbit synovium expresses predominantly HAS2 (Momberger *et al.* 2005). HA elongation takes place at the intracellular reducing end of the chain by alternate UDP-sugar addition, whilst the non-reducing end is simultaneously extruded

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through a trans-membrane pore into the extracellular space (Bodevin-Authelet *et al.* 2005). HA is not stored in secretory granules but is released directly from the HAS extracellular domain, so any increase in HA secretion requires increased net HAS activity.

The regulation of HA secretion rate has been studied mainly in non-synovial cell lines *in vitro*. Secretion can be stimulated over 24 h by interleukin 1β, transforming growth factor TGFβ, and platelet-derived growth factor. This activates the phospholipase C–diacyl glycerol–protein kinase C (PKC)–ERK1/2 signal pathway leading to protein synthesis, possibly HAS (Heldin *et al.* 1992; Klewes & Prehm, 1994; Haubeck *et al.* 1995; Pienimäki *et al.* 2001). In rabbit knees phorbol ester-activated PKC stimulates HA secretion by $\geq$ 100% (Anggiansah *et al.* 2003). Moreover static stretch of rabbit intimal synoviocytes activates a Ca^{2+}-sensitive PKCα–ERK1/2 pathway that stimulates HA secretion by 57% over 3 h (Momberger *et al.* 2005, 2006), while cyclic stretch stimulates HA secretion by cultured chick embryo fibrocartilage cells (Dowthwaite *et al.* 1999, 2003) and cervical fibroblasts (Takemura *et al.* 2005). Static stretch and HA dilution stimulate HA secretion in intact rabbit knee joints, into both the synovial extracellular matrix (Price *et al.* 1996*b*) and the joint cavity (25% increase in 4 h, Coleman *et al.* 1997). An increase in HA production in response to washout has been noted in skin, intestine and lung, e.g. Townsley *et al.* (1994). The secretory response appears to serve a homeostatic role, because it helps to prevent the HA concentration from falling in response to diluting influences.

Previous synovial studies have reported only the effect of *static* stretch, whereas the key role of joints is movement. Since HA protects the joint against the potentially damaging effects of repetitive joint movement, we formed the hypothesis that HA secretion may be physiologically coupled to joint movement, and that dynamic joint motion may stimulate HA secretion more than static distension. Moreover exercise therapy, like intra-articular HA therapy, can provide symptomatic relief in moderate osteoarthritis and improve synovial fluid viscosity (van Baar *et al.* 1999; Miyaguchi *et al.* 2003). The proposed coupling of HA secretion to movement could help explain this and provide a link between the medical benefits of intra-articular HA therapy and exercise therapy. Our primary aim therefore was to test the hypothesis that cyclic joint movement stimulates HA secretion *in vivo*. We also evaluated the washout methodology and its theoretical basis, assessed the possible inflammatory effect of joint cannulation, examined the metabolic requirements for HA production *in vivo*, and measured trans-synovial HA loss.

Methods

Hyaluronan secretion was measured in static and cycled rabbit knees over 5 h using an optimized washout method. The recovered HA was analysed by high performance gel exclusion chromatography (HPLC). A paired experimental design was used in many studies. One knee was subjected to the treatment under investigation, e.g. movement or an intra-articular injection of active agent such as a metabolic blocker, as specified in Results. The opposite knee was subjected to an appropriate control treatment, e.g. no movement or an intra-articular injection of vehicle without active agent. In addition secretion was measured in many unpaired static or moved joints over the course of a continuing programme to study secretion regulation.

Materials

The synovial cavity was washed out with a sterile, non-pyrogenic salt solution (147 mM Na^+, 4 mM K^+, 2 mM Ca^{2+}, 155 mM Cl^-; Steriflex Ringer solution, Fresenius Kabi Ltd, Warrington, UK). Active agents were from Sigma-Aldrich (Poole, UK).

Animal preparation

New Zealand White rabbits weighing 2.0–3.8 kg (mean $\pm$ S.E.M. 2.93 $\pm$ 0.02 kg) were anaesthetized by I.V. sodium pentobarbitone (30 mg kg^{-1}) (Pentoject, York, UK) and ethyl carbamate (urethane, 500 mg kg^{-1}). Inhibition of the corneal blink reflex and paw pinch reflex was maintained by repeat doses every 30 min. After tracheostomy and pump ventilation, the extended knee joint cavity (100–130 deg) was cannulated lateral to the patellar ligament by a sterile, non-pyrogenic 20 gauge Medicut cannula (Sherwood Medical, Tullamore, Republic of Ireland) secured by a purse-string suture. The study conformed to UK 1986 animal legislation and local animal welfare committee guidelines. At the end of the study ($\sim$5 h) the animal was killed by an overdose of I.V. sodium pentobarbitone (2 ml Euthatal, Rhône Mérieux Ltd, Harlow, UK).

Recovery of endogenous hyaluronan from synovial cavity

Endogenous HA was washed out of the synovial cavity prior to the secretion study. Ringer solution (1 ml, 25–37°C) was injected into the synovial cavity over a timed 8 s interval. The joint was flexed and extended 5 times at 0.5 Hz to mix the injectate with the highly viscous synovial fluid. The wash fluid was then aspirated, aided by digital pressure, over the popliteal fossa. The washout with 1 ml fresh Ringer solution was repeated 18 times. Hyaluronan recovery usually fell below a measurable level ($\sim$3 μg ml^{-1}) in the last few washes. An algebraic analysis of the slow washout process is given in the

Appendix. Washouts were performed simultaneously on the two knee joints by two operators, the operator side being randomized. The washes were stored at −80°C until analysis.

Previous washout methods have varied considerably (Denlinger, 1982; Brown *et al.* 1991; Coleman *et al.* 1997). We therefore evaluated the method variables systematically to determine the optimum mixing cycles per wash to maximize recovery, optimal wash volume V_w and wash temperature. The above protocol is based on the results, which are presented as online Supplemental material.

Movement protocol and recovery of newly secreted hyaluronan

After the 18th wash the joint was subjected to passive cyclic movement or remained static in 100–130 degrees extension. The knee joint was flexed and extended by hand, holding the hind limb paw. Movement spanned the full, unforced range at a metronome-regulated rate of 0.5 Hz for 3 min followed by 12 min rest, since rabbits naturally exhibit an intermittent movement pattern. This procedure was continued for 5 h, during which interval the synovium secreted > 10 times the minimum detectable HA mass. The joint was then washed out by 15 × 1 ml Ringer washes, with five mixing cycles per wash, to harvest the newly produced HA.

Sample preparation and analysis

Sample treatment and analysis are detailed in Supplemental material. Briefly, the recovered washes were centrifuged (7800 *g*, 10 min), pooled (3–5 ml) and papain-digested (5.6 units at 60–65°C for 1 h) to remove a prominent albumin band. Papain digestion does not affect HA molecular mass (Coleman *et al.* 1997). HA quantity and molecular size were analysed by size exclusion, high performance liquid chromatography (HPLC) (Waters Ltd, Watford, UK), using a TosoHaas TSK G6000 PW_{XL} column (Anachem Ltd, Luton, UK) and ultraviolet absorbance at 206 nm. Concentration calibration curves for 2000 kDa rooster comb HA were linear from 3 μg ml^{-1} to 200 μg ml^{-1}. Curves relating retention time (t_{ret}) to HA mol.wt have been published previously (Coleman *et al.* 1997). The apparent rate of secretion of HA, $\dot{q}_{HA}$, i.e. the net accumulation rate uncorrected for trans-synovial loss, was calculated as the total mass of HA recovered in the second set of washes divided by secretion time.

Tests of active secretion

Four paired-design studies with metabolic inhibitors were undertaken to confirm active secretion and exclude passive leaching of pre-existing peri-articular HA into the cavity. (1) Formaldehyde (3.7–7.4%) was injected into the joint cavity prior to secretion to cross-link synoviocyte enzymes. (2) Joints were pre-treated with intra-articular sodium iodoacetate (10 mM), which alkylates sulphydryl (thiol) groups to inactivate cysteine-containing enzymes such as phosphoglyceraldehyde dehydrogenase and coenzyme A, key enzymes in the glycolytic energy supply for HA synthesis. The inactivation of cysteine moieties in HAS reduces its activity to < 10% (Pummill & DeAngelis, 2002), so iodoacetate may also reduce HAS activity directly. (3) Intra-articular 2-deoxyglucose (83 mM, total osmolarity of injectate 283 mosmol l^{-1}) competitively inhibited hexokinase (glucose → glucose-6-phosphate) and hence glucose entry into the HA synthetic pathway (IC_{50} 4 mM, relative affinity 0.15–0.6, Hashimoto *et al.* 1999). (4) Sodium 4-methylumbelliferone (5 mM) is a proposed competitive substrate for UDP-glucosyltransferase, leading to a fall in UDP-glucuronate supply (Kakizaki *et al.* 2004) and/or an inhibitor of HAS expression (Rilla *et al.* 2004; Kultti *et al.* 2005). Active agents were injected into the joint cavity in a 0.5–1 ml bolus at 5–15 min prior to the secretion period. Since small, rapidly diffusible solutes clear rapidly from the joint cavity, an intra-articular top-up dose of 0.2–0.3 ml agent or Ringer solution (control) was injected every 30 min. Each drug injection was followed by five slow mixing cycles.

Glucose availability in washed joints

Glucose, the primary building-block of hyaluronan, reaches synoviocytes by diffusion from fenestrated capillaries just a few micrometres below the synovial surface (Levick & McDonald, 1989). To assess glucose availability after joint washout, the 18th wash and samples of intra-articular fluid taken 60 min, 150 min and 300 min later were analysed using a glucose oxidase-based meter (Accu-check Advantage, Mannheim, Germany) calibrated by known D-glucose concentrations.

Tests for stimulation or inflammation by intra-articular cannula

Three studies based on the paired-experiment design investigated whether mechanical stimulation and/or inflammation by the intra-articular cannula contributed to the response. (1) Synovial inflammation was assessed in moved, cannulated joints by the permeation of intravenously injected Evans blue-labelled albumin (EVA; Evans blue : bovine serum albumin ratio 1 : 12) into the joint cavity (Poli *et al.* 2002). After the initial HA washouts 10 ml EVA was injected into the marginal ear vein at time zero and its appearance in the joint cavity monitored by washout. (2) The effect of movement was measured in

joints treated with intra-articular indomethacin (14 μM), a potent inhibitor of cyclo-oxygenase and inflammatory prostaglandin production used to treat human arthritis. (3) Experiments were carried out with the cannula withdrawn from the cavity throughout the 5 h period of movement.

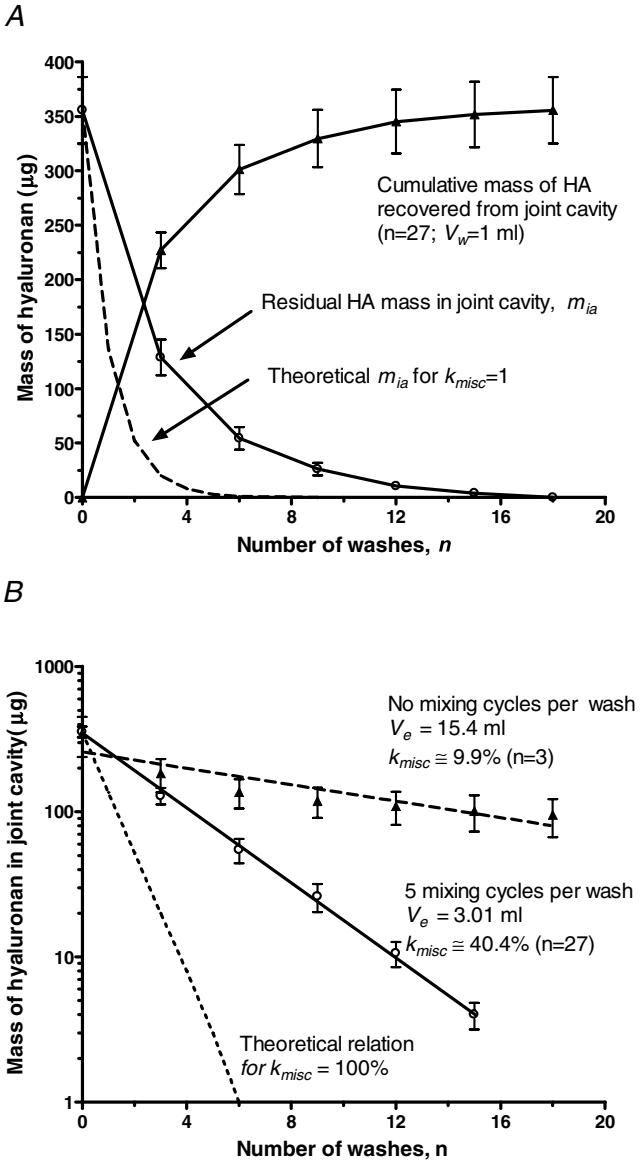

Figure 1. Washout of hyaluronan (HA) from joint cavity and estimation of intra-articular miscibility
A, cumulative recovered HA mass and residual mass in joint cavity after each intra-articular wash for 18 washes of 1 ml Ringer solution (V_w) with 5 mixing cycles per wash (mean ± S.D., $n = 27$ joints). Dashed line shows rate of washout expected if HA mixed perfectly with each 1 ml injectate (Appendix eqn (1) for $k_{misc} = 1$; $V_r = 0.62$ ml). *B*, linearization of residual mass curve by logarithmic plot (eqns (2) and (3)). The miscibility coefficient k_{misc} calculated from the regression slope s was 0.404 (40%) at 5 mixing cycles wash^{-1} and fell to 0.099 (9.9%) at zero mixing cycles, demonstrating the necessity for mixing cycles. Due to the low miscibility of HA, the equivalent residual volume V_e greatly exceeds the physical residual volume V_r of 0.62 ml (Appendix eqn (2)).

Estimation of fractional HA loss from joint cavity over 5 h

The net accumulation of HA over 5 h may underestimate the true intra-articular secretion rate, because HA escapes slowly through the synovial lining despite substantial reflection (Sabaratnam *et al.* 2004). To assess the fractional loss endogenous HA was removed by washout and the joint injected every 30 min with a 0.3 ml solution containing ∼214 μg rooster comb HA (ROCHA) or ∼170 μg endogenous rabbit synovial HA (ERSHA), to simulate the progressive accumulation of HA over 5 h. The total amount injected, I, was 1900–1922 μg of rooster comb HA (ROCHA, ∼2000 kDa, Sigma) or 1488–1753 μg of endogenous rabbit synovial fluid HA (ERSHA, ∼3500 kDa). The large injected mass reduced the fractional contribution of secreted HA to < 7%. The ERSHA was obtained by freeze-drying the endogenous HA recovered from many rabbit knees. At 5 h the cavity was washed out and the amount of HA remaining in the cavity (R) was analysed.

The HA loss estimated by the above method is a slight underestimate due to intra-articular secretion, just as the apparent secretion rate $\dot{q}_{HA}$ is an underestimate due to losses. The solution of the simultaneous equations for these processes over 5 h gives the true secretion rate $\dot{q}_{HA}^{corrected} = \dot{q}_{HA} I/(R - 5\dot{q}_{HA})$ and the true loss $L = (I + 5\,\dot{q}_{HA}^{corrected}) - R$.

Statistical analysis

Means ± S.E.M. are cited throughout. Normality was assessed by the D'Agostino–Pearson test and results compared by Student's t test or Mann–Whitney U test. Regression analysis, Pearson's correlation coefficient r and slope comparison by analysis of covariance (ANCOVA) were as implemented in Graphpad Prism (San Diego, CA, USA). Significance was accepted at $P \leq 0.05$.

Results

HA washout rate, residual volume and miscibility coefficient

The cumulative recovery of endogenous HA is shown in Fig. 1*A*. The final wash content, 4 μg, was near the limit of resolution and just 1.2% of the total. The residual intra-articular mass m_{ia} after n washes (total recovered mass minus cumulative mass at wash n) was a log–linear function of n (Fig. 1*B*) as predicted by washout theory (Appendix eqns (2) and (3)). The removal rate constant (regression slope s) was -0.287 ± 0.008 n^{-1}, showing that only 28.7% of the remaining intra-articular HA was removed by each wash – much less than expected for a freely miscible solute. Substitution of s into Appendix

eqn (4a) gave an equivalent residual volume V_e of 3.01 ml, which is ~5 times the actual residual volume V_r, ~0.62 ml (cannula dead space 0.22 ml, residual intra-articular fluid volume 0.4 ml at 1 cmH_2O; Knight & Levick, 1982). This showed that endogenous HA mixed only partially with the Ringer solution during each wash. The miscibility coefficient k_{misc} (eqn (4b)) was 0.404, i.e. only ~40% of the HA left in the joint cavity passed into the Ringer solution during each washout.

Endogenous HA mass and volume of synovial fluid

The endogenous HA mass m_{endog} was $325 \pm 7\ \mu g$ per joint ($n = 435$) and the residual unrecovered mass was $\sim 2\ \mu g$ (Appendix eqn (2), 99.4% recovery, $n = 18$ washes, $V_e = 3.01$ ml). Residual HA thus contributed little to the 55–110 μg of HA found in the joint at 5 h. The HPLC retention time t_{ret} of the endogenous HA, 6.757 ± 0.01 min, indicated a molecular mass of ~3500 kDa. The volume of native synovial fluid was 90 μl, calculated as mean HA mass/mean HA concentration in undiluted native fluid samples (3.6 mg ml^{-1}, Price *et al.* 1996*a*).

Since a control joint was often compared with the paired, contralateral test joint, we tested the inherent assumption of side-to-side symmetry by comparing m_{endog} in paired joints (Fig. 2*A*). Results for the two sides correlated well ($r = 0.80$, $P < 0.0001$) and the slope of the relation, 0.76 ± 0.05, was not significantly different from equality.

Since m_{endog} spanned a wide range (25th to 75th percentile 207–421 μg), we assessed the contribution of the variation in rabbit size (range 2.0–3.8 kg, mean 2.93 kg). Rabbit weight and m_{endog} showed a significant but low correlation ($r = 0.27$, $P < 0.0001$), indicating that additional factors influenced m_{endog} (Fig. 2*B*). To reduce the effect of weight variation, m_{endog} and secretion rates were normalized to a standard 3 kg rabbit by weight ratio. This reduced the coefficient of variation from 47% to 41%. The weight-normalized distribution of m_{endog} was skewed, with a mode of 300 μg and mean $367 \pm 8\ \mu g$ ($n = 335$) (Fig. 2*C*).

HA secretion and molecular size in static joints

The rate of HA accumulation over 5 h in static joints, $\dot{q}_{HA}$, was $10.9 \pm 0.7\ \mu g\,h^{-1}$ ($n = 90$), or $11.2 \pm 0.7\ \mu g\,h^{-1}$ normalized to a 3 kg animal ($\dot{q}_{HA3}$). As with m_{endog}, $\dot{q}_{HA3}$ spanned a wide range (25th to 75th percentile 7.0–13.7 $\mu g\,h^{-1}$, coefficient of variation 59%) (Fig. 3*A*). Although $\dot{q}_{HA}$ did not correlate significantly with weight ($r = 0.13$, $P = 0.22$), it correlated strongly with m_{endog} ($r = 0.70$, regression slope $0.029 \pm 0.003\ h^{-1}$, $P < 0.0001$). Thus joints with large secretion rates had large endogenous HA masses. This finding is important because it indicates that the wide range of m_{endog} and $\dot{q}_{HA}$ is a feature of synovial biology rather than methodological error.

In view of the above correlation, and to facilitate the comparison of results between non-paired studies where endogenous mass differed, $\dot{q}_{HA}$ was normalized per 100 μg endogenous HA ($\%\dot{q}_{HA}$). This reduced the coefficient of variation from 59.2% to 44.6%, the skew from 2.3 to 1.3 and the kurtosis from 9.2 to 2.2 (Fig. 3*B*). Mean $\%\dot{q}_{HA}$ was $3.43 \pm 0.16\ \mu g\,h^{-1}\ (100\ \mu g)^{-1}$ ($n = 90$), i.e. secretion replaced at least 3.4% of the endogenous mass per hour.

The molecular size of the newly secreted HA (t_{ret} 6.719 ± 0.023 min, $n = 90$) correlated closely with that of the paired endogenous HA (t_{ret} 6.712 ± 0.022 min, $n = 90$, $r = 0.975$, $P < 0.0001$). The difference was not significant ($P = 0.75$, Student's paired t test).

In some static studies the joint was undisturbed for 5 h, while in other static joints (controls in paired studies) 0.3 ml Ringer was injected every 30 min with 5 mixing cycles. The secretion rate $\dot{q}_{HA3}$ in undisturbed joints, $11.4 \pm 1.0\ \mu g\,h^{-1}$ ($n = 35$), was not significantly different from $\dot{q}_{HA3}$ in the Ringer-injected joints, $10.5 \pm 0.9\ \mu g\,h^{-1}$ ($n = 55$) ($P = 0.49$, Student's unpaired t test). This observation is important because it shows that brief episodes of fluid shear stress and movement did not stimulate HA secretion significantly in the joints designated as static.

Stimulation of HA secretion by cyclic movement

In 35 paired-design experiments one joint remained static and the contralateral joint was moved cyclically, without further treatment. Movement increased the net secretion rate $\dot{q}_{HA3}$ in 34/35 cases (Fig. 4*A*). The increase averaged 91%, from $10.3 \pm 0.9\ \mu g\,h^{-1}$ (static) to $19.7 \pm 1.5\ \mu g\,h^{-1}$ (moved) ($P < 0.0001$, Student's paired t test). The movement-stimulated $\dot{q}_{HA3}$ correlated strongly with the contralateral static $\dot{q}_{HA3}$ ($r = 0.82$, $P < 0.0001$), i.e. the largest movement-stimulated secretion rates occurred in the animals with the largest static secretion rates (Fig. 4*C*). The increase in $\dot{q}_{HA3}$ also correlated with m_{endog} in the same joint ($r = 0.67$, $P < 0.0001$) (Fig. 4*D*). The normalized secretion rate $\%\dot{q}_{HA}$ increased from $3.69 \pm 0.30\ \mu g\,h^{-1}\ (100\ \mu g)^{-1}$ in the static joints to $6.43 \pm 0.39\ \mu g\,h^{-1}\ (100\ \mu g)^{-1}$ in paired moved joints.

In addition to the 35 paired static-*versus*-moved experiments, secretion was measured in many additional moved joints accumulated over the course of a larger, ongoing study of secretion regulation. Mean $\dot{q}_{HA3}$ was $22.6 \pm 1.2\ \mu g\,h^{-1}$ ($\dot{q}_{HA}$ $22.0 \pm 1.2\ \mu g\,h^{-1}$) in 77 cyclically moved joints, double the secretion rate $\dot{q}_{HA3}$ in 90 static joints, $11.2 \pm 0.7\ \mu g\,h^{-1}$ ($P < 0.0001$, Mann–Whitney U test) (Fig. 4*B*). The normalized secretion rate, $\%\dot{q}_{HA}$, was likewise much higher in moved joints,

$5.92 \pm 0.27\ \mu g\ h^{-1}\ (100\ \mu g)^{-1}$ ($n = 77$) than in static joints, $3.43 \pm 0.16\ \mu g\ h^{-1}\ (100\ \mu g)^{-1}$ ($n = 90$) (Fig. 3*B*). The movement-stimulated secretion rates spanned a wide range and correlated strongly with the endogenous mass ($r = 0.66$, slope $0.044 \pm 0.006\ h^{-1}$, $P < 0.0001$, $n = 77$). Thus the joints with the largest m_{endog} tended to have the largest static secretion rate and also the largest response to movement.

The molecular size of HA secreted by moved joints, as assessed by t_{ret}, was the same as in the paired, static joints (6.738 ± 0.042 min, 6.733 ± 0.041 min, respectively, $P = 0.73$, $n = 35$, Student's paired t test). Similarly, t_{ret}

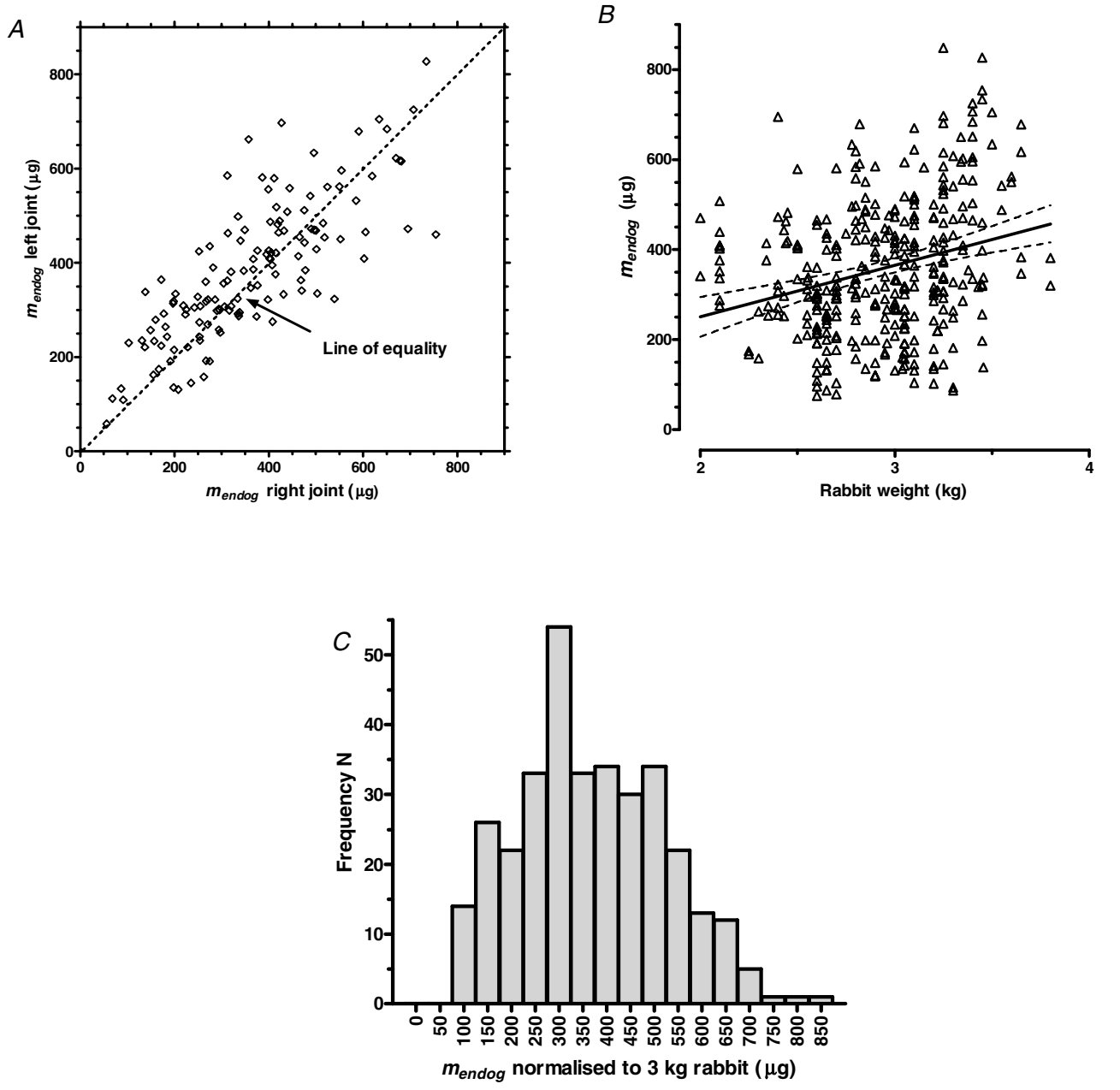

Figure 2. Mass of endogenous hyaluronan (m_{endog}) in synovial cavity, demonstrating side-to-side symmetry and variation with body weight

A, correlation between m_{endog} recovered from left and right knee joints of 134 rabbits (Pearson's $r = 0.80$, $P < 0.0001$). Coefficient of variation 47%. *B*, relation between m_{endog} and animal weight; regression line with 95% confidence bands. The correlation was significant but weak (Pearson's $r = 0.27$, $P < 0.0001$, $n = 340$). *C*, distribution of m_{endog} normalized for a 3 kg rabbit by weight ratio ($n = 335$). The distribution is skewed (Kolmogorov–Smirnov normality test $P = 0.01$), with a mode of 300 μg, mean $367 \pm 8.3\ \mu g$ (S.E.M.), coefficient of variation 41%.

for HA secreted by moved joints was not significantly different from the paired endogenous HA t_{ret}; the two parameters correlated closely ($r = 0.94$, $P < 0.0001$, $n = 72$) and a plot of moved t_{ret} *versus* endogenous t_{ret} had a slope close to unity, 0.95 ± 0.04 (Fig. 5).

Tests of active secretion; effect of metabolic inhibitors

The amount of HA appearing in the joint cavity over 5 h greatly exceeds the amount in synovium (Price *et al.* 1996*a*), so passive leaching of pre-formed synovial HA seems an unlikely source. Metabolic inhibitors were used to confirm directly that the accumulated HA resulted from active cellular secretion, and also to test the role of glucose metabolism in HA secretion. These studies followed the paired design outlined in Methods.

Formaldehyde, which inactivates intracellular enzymes by cross-linkage, almost totally blocked movement-stimulated HA secretion ($n = 6$ pairs of joints, Fig. 6). The secretion rate in formaldehyde-treated moved joints was 7% of that in paired, static Ringer-treated controls (Table 1). The true secretion rate was probably even closer to zero, since the residual HA after the initial washout of m_{endog} (447 μg), namely 2.6 μg (Appendix eqn (2)), would simulate an apparent secretion rate of 0.5 $\mu g\,h^{-1}$.

Iodoacetate treatment reduced $\dot{q}_{HA3}$ in moved joints to 4% of that in the control moved joint ($n = 5$, Fig. 6), and reduced $\dot{q}_{HA3}$ in static joints to 14.7% ($n = 5$, Table 1). Iodoacetate-treated joints also showed an increase in HA loss (see later), but this did not fully explain the 24-fold fall in moved $\dot{q}_{HA3}$. The ability of iodoacetate to inhibit HA secretion was confirmed *in vitro* in a collaborative pilot study with Dr A. Pitsillides, Dr C. Clarkin and B. Wheeler (Royal Veterinary College, London). HA secretion by cultured chick embryo fibrocartilage cells over 6 h was 2.14 ± 0.45 ng (mg protein)$^{-1}$ in control cultures ($n = 3$) and was unaffected (105% control) by 10 μM iodoacetate ($n = 3$), but was reduced to 4.5% of control by 1 mM iodoacetate ($n = 3$) and zero by 10 mM iodoacetate ($n = 3$).

2-Deoxyglucose (2DG) treatment reduced the HA secretion rate to 44.5% of the control level in moved joints ($n = 6$, Fig. 6) and to 20.4% in static joints ($n = 5$, Table 1). Inhibition was confirmed *in vitro* in collaboration with Dr Pitsillides and colleagues (see above); 83 mM 2DG reduced HA secretion over 6 h to zero ($n = 3$), with no evidence of cellular damage. The results show that HA production *in vivo* and *in vitro* depends substantially on the continuous cellular uptake and hexokinase-mediated metabolism of glucose rather than stored glycogen.

4-Methylumbelliferone (4MU) treatment had a variable effect ($n = 9$, Fig. 6). The small fall in moved secretion rate, to 87% of control, was not significant

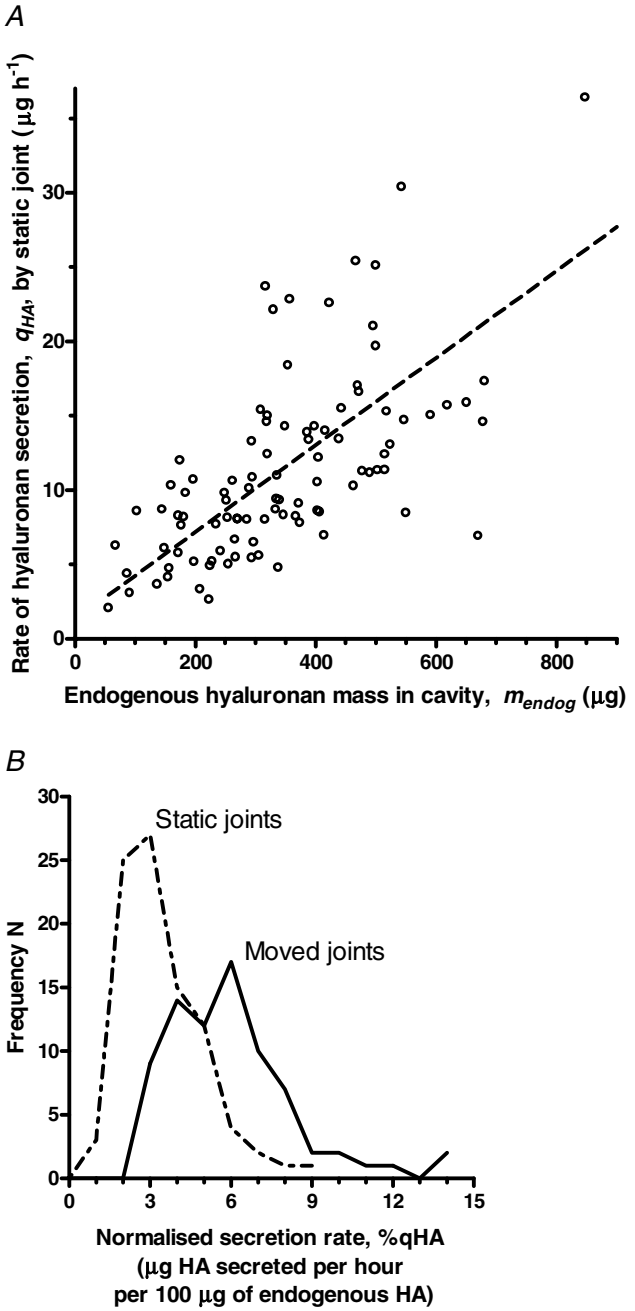

Figure 3. Net rate of hyaluronan accumulation $\dot{q}_{HA}$ in cavity of rabbit knee joint
A, the net secretion rate in static joints correlated well with the amount of hyaluronan in the synovial cavity prior to washout, m_{endog} (regression slope 0.0293 ± 0.0031 $\mu g\,h^{-1}$ (100 μg)$^{-1}$ or 2.9% h^{-1}, $P < 0.0001$). Mean static $\dot{q}_{HA}$ was 10.9 ± 0.7 $\mu g\,h^{-1}$ ($n = 90$). *B*, net secretion rate normalized per 100 μg of endogenous mass (%$\dot{q}_{HA}$), i.e. the percentage of the endogenous HA replaced by secretion each hour. Normalization reduced the coefficient of variation from 58.2% ($\dot{q}_{HA}$) to 44.6% (%$\dot{q}_{HA}$) in static joints (dashed line). The distribution of static %$\dot{q}_{HA}$ is skewed (Kolmogorov-Smirnov normality test $P = 0.003$), with a mode of 3.0% h^{-1} and mean 3.43 ± 0.16% h^{-1} ($n = 90$). Movement shifted the distribution of %$\dot{q}_{HA}$ to high values (mode 6.0% h^{-1}, mean 5.9 ± 0.3% h^{-1}, $n = 77$) (continuous line).

(Table 1). 4MU did not reduce $\dot{q}_{HA3}$ in two pairs of static joints (changes $+1\ \mu g\,h^{-1}$, $+5\ \mu g\,h^{-1}$).

Precursor availability in washed joints

The initial washouts with glucose-free Ringer solution reduced the intra-articular glucose to 5.9 mM. The corresponding blood glucose was 11.5 mM. The subsequent intra-articular glucose at 60 min, 150 min and 300 min, measured in samples taken 30 min after 0.3 ml glucose-free Ringer top-ups in control studies, averaged 11.6 ± 0.9 mM ($n = 5$). Glucose was thus freely available to the synoviocytes. Glutamine (146 Da), the other requirement for glucosamine synthesis, is present in rabbit plasma at 0.5 mM (Rapoport *et al.* 1971) and has a similar diffusivity to glucose.

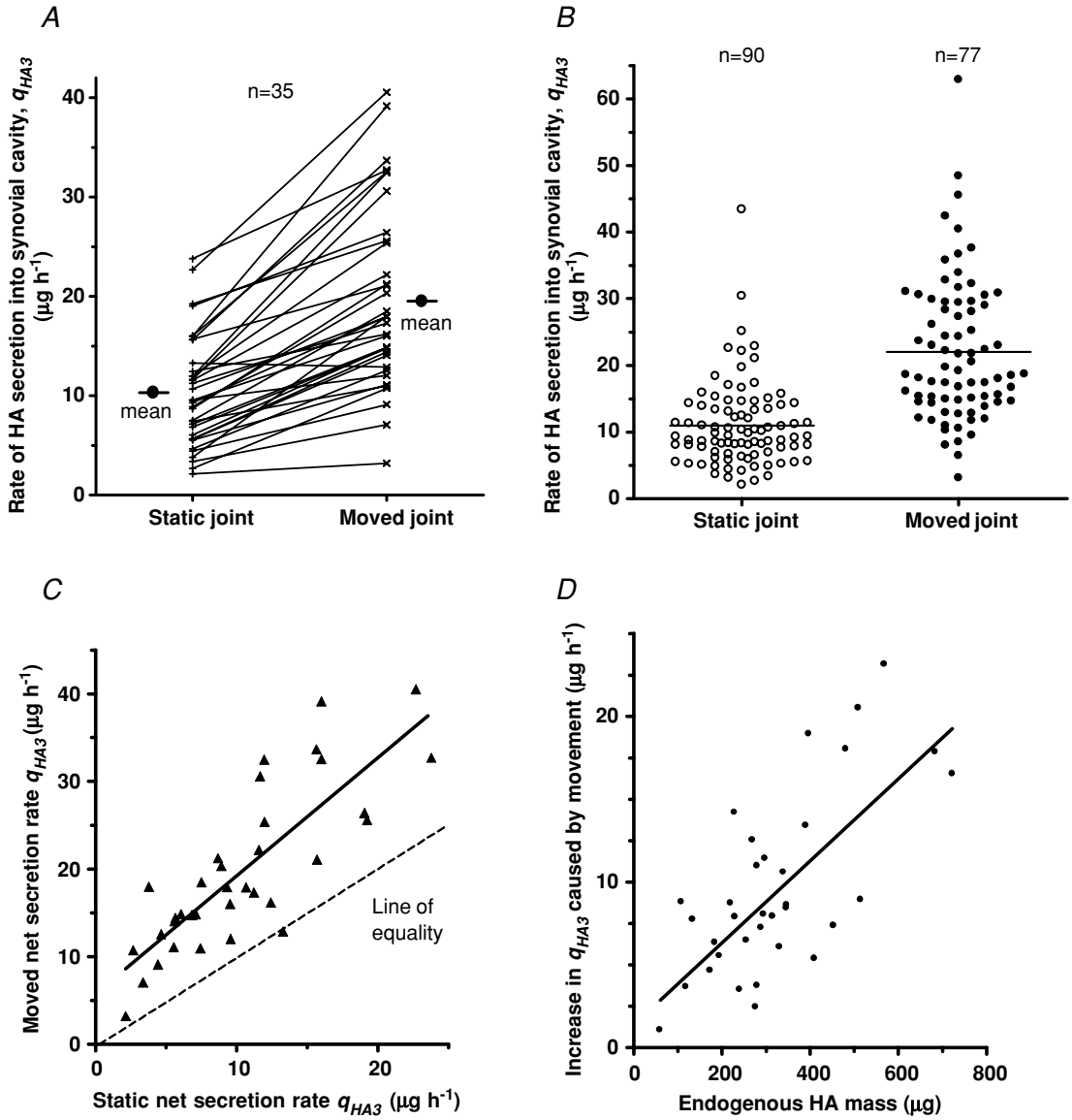

Figure 4. Effect of cyclic movement on hyaluronan secretion
Passive, full-range cycles at 0.5 Hz were carried out for 3 min periods alternating with 12 min rest over 5 h. *A*, paired experiments with one joint static and the contralateral joint moved. Movement raised $\dot{q}_{HA3}$ in 34/35 animals, by 91% on average ($P < 0.0001$, paired *t* test). *B*, unpaired comparison of $\dot{q}_{HA3}$ in 90 static joints and 77 moved joints. Movement doubled the secretion rate from 11.2 ± 0.7 to $22.6 \pm 1.2\ \mu g\,h^{-1}$ ($P < 0.0001$, Mann–Whitney *U* test). *C*, the effect of movement was greatest in joints with the high static secretion rates (regression slope 1.35 ± 0.17, $r = 0.82$, $P < 0.0001$). The absolute increase in secretion rate (the difference) likewise correlated with static secretion rate ($r = 0.34$, $P = 0.04$). *D*, the increase in secretion rate with movement also correlated with the endogenous mass of HA in the joint cavity prior to movement (slope $0.025 \pm 0.005\ h^{-1}$, $r = 0.67$. $P < 0.0001$).

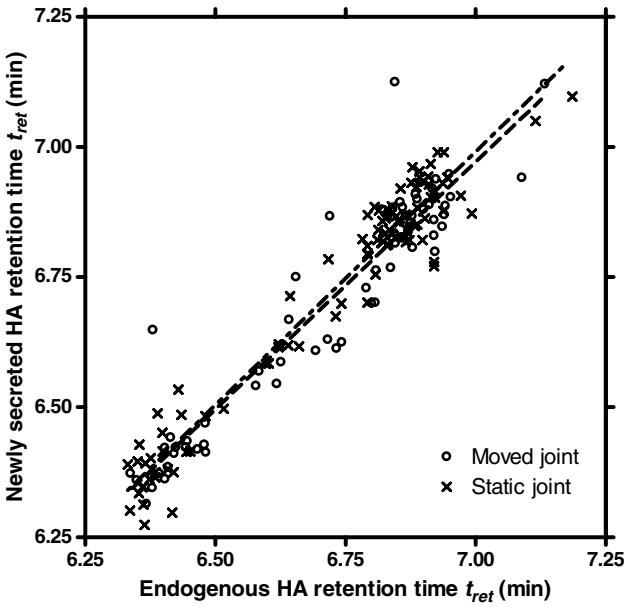

Figure 5. Molecular size of newly secreted hyaluronan compared with hyaluronan in native synovial fluid from the same joint
Molecular size was determined as HA retention time t_{ret} during size-exclusion chromatography. Most results are close to the line of equality, both for static joints (crosses) and moved joints (circles). The slope of the regression line for static joints (dot–dashed line, 0.97 ± 0.02, $n = 90$) or moved joints (dashed line, 0.95 ± 0.04, $n = 72$) was not significantly different from 1 (equality), and the correlation coefficient r was, respectively, 0.97 and 0.94. The clustering is caused by a change of HPLC column, with ~0.5 min difference in t_{ret} between columns for a standard sample.

Tests for stimulation/inflammation by the intra-articular cannula

Plasma albumin permeation into joint cavity. Protein clearance from plasma into the synovial cavity was determined by the colorimetric assay of joint washouts for EVA at 6 h. In three pairs of joints one joint remained static and the other was moved, both with the cannula *in situ* throughout. Plasma albumin clearance was low, namely $7.7 \pm 3.8\,\mu l\,h^{-1}$ in the static joints and $8.3 \pm 2.7\,\mu l\,h^{-1}$ in the contralateral moved joints ($P = 0.7$, $n = 3$, Student's paired t test). The clearance is normally $6.2 \pm 1.4\,\mu l\,h^{-1}$ and increases 25-fold during inflammation (Poli *et al.* 2002). There was thus no evidence of significant inflammation during movement with the cannula *in situ*.

Cyclo-oxygenase inhibition. In three pairs of cannulated joints one joint remained static and the other was moved; both were treated with intra-articular indomethacin. Indomethacin treatment did not inhibit either the movement-stimulated or static secretion rates; the $\dot{q}_{HA3}$ doubled with movement from $10.8 \pm 0.7\,\mu g\,h^{-1}$ in the static, treated joints to $21.6 \pm 4.9\,\mu g\,h^{-1}$ in the moved, treated joints ($n = 3$).

Cannula removal. In 13 paired experiments the intra-articular cannula was removed from one of the joints after the initial washouts and re-inserted at 5 h to harvest the secreted HA. In eight of these experiments the cannula-free joint was moved for 5 h while the contralateral cannulated joint was static. Movement

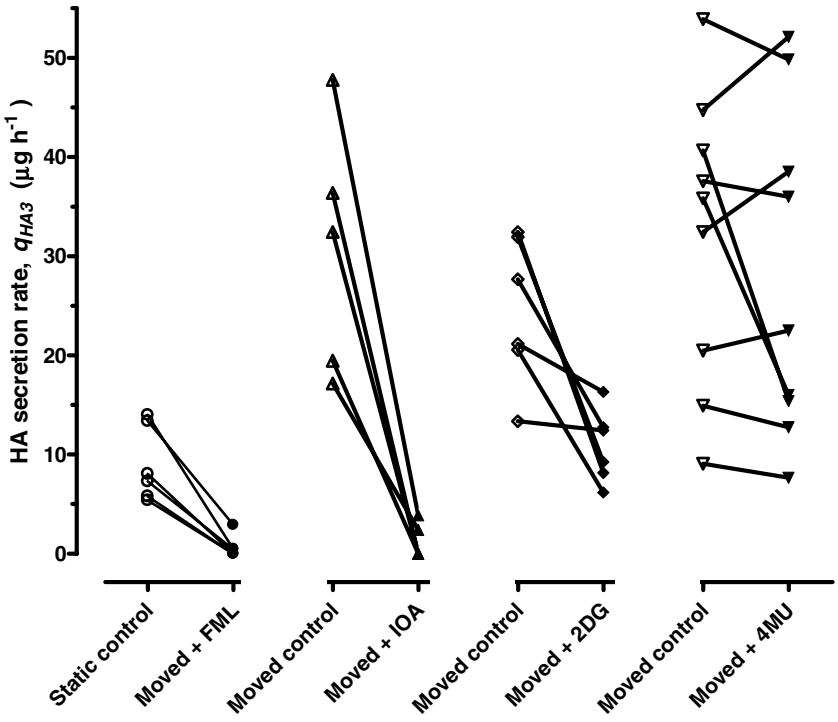

Figure 6. Effect of intra-articular injection of metabolic inhibitors on hyaluronan harvest
HA was harvested from the joint cavity after a 5 h secretion period. Metabolic inhibitors were injected into the joint cavity just prior to the secretion period and thereafter at 30 min intervals (see Methods). Control joints received matching volumes of Ringer solution. FML, formaldehyde (3.7–7.4%). IOA, sodium iodoacetate (10 mM). 2DG, 2-deoxyglucose (83 mM). 4MU, sodium 4-methylumbelliferone (5 mM).

Table 1. Paired studies of effect of metabolic inhibitors on synovial HA secretion rate *in vivo*

Treatment	Joint stimulus	$\dot{q}_{HA3}$ (μg h^{-1})	$\%\dot{q}_{HA}$ (μg h^{-1} (100 μg)$^{-1}$)	*n*	*P**
Vehicle (control side)	Static	9.5 ± 1.8	2.8 ± 0.37	6	0.002
Formaldehyde	Moved	0.7 ± 0.5	0.15 ± 0.11		
Vehicle (control side)	Moved	30.7 ± 5.6	5.8 ± 0.7	5	0.002
Iodoacetate	Moved	1.3 ± 0.8	0.3 ± 0.2		
Vehicle (control side)	Static	14.9 ± 2.5	2.4 ± 0.3	5	0.004
Iodoacetate	Static	2.2 ± 0.6	0.43 ± 0.14		
Vehicle (control side)	Moved	24.5 ± 3.1	6.0 ± 0.6	6	< 0.001
2-Deoxyglucose	Moved	10.9 ± 1.5	2.3 ± 0.4		
Vehicle (control side)	Static	26.0 ± 3.1	5.0 ± 0.8	5	0.002
2-Deoxyglucose	Static	5.3 ± 0.6	0.95 ± 0.11		
Vehicle (control side)	Moved	32.2 ± 4.9	5.83 ± 0.53	9	0.28
4-Methylumbelliferone	Moved	27.9 ± 5.5	5.43 ± 0.81		

$\dot{q}_{HA3}$ and $\%\dot{q}_{HA}$ values are mean ± S.E.M. * Student's paired *t* test for $\%\dot{q}_{HA}$; test outcomes for $\dot{q}_{HA3}$ are not materially different.

almost doubled $\dot{q}_{HA3}$ in the cannula-free joints, from 12.9 ± 2.0 μg h^{-1} (static control) to 24.1 ± 4.7 μg h^{-1} ($P = 0.018$, Student's paired *t* test). In the other five paired experiments one joint was moved after cannula removal and the contralateral joint was moved with the cannula *in situ*. Cannula removal did not significantly alter the movement-stimulated $\dot{q}_{HA3}$ (30.3 ± 1.1 μg h^{-1} without cannula, 32.8 ± 1.9 μg h^{-1} with cannula; $P = 0.41$, Student's paired *t* test).

HA loss from joint cavity over 5 h

Following the cumulative injection of exogenous ROCHA (1900–1922 μg) or ERSHA (1488–1753 μg) over 5 h, 53.6 ± 6.0% ($n = 5$) of the injected ROCHA was recovered from static joints and 55.2 ± 3.6% ($n = 5$) of the injected ERSHA. In moved joints 60.5 ± 4.6% ($n = 5$) of the injected ROCHA was recovered and 57.6 ± 6.1% ($n = 6$) of the injected ERSHA. The rate of loss of injected HA was thus 8–9% h^{-1}. In view of the cytotoxicity of iodoacetate, its effect on HA retention was assessed in the contralateral joints in some of the above experiments. In static joints iodoacetate reduced ROCHA recovery to 24.0 ± 5.6% ($n = 5$) and ERSHA recovery to 32.3 ± 7.4% ($n = 3$). In moved joints iodoacetate reduced ROCHA recovery to 14.3 ± 2.7% ($n = 5$). These effects, though substantial, are insufficient to explain the much greater inhibition of $\dot{q}_{HA}$ by iodoacetate (Fig. 6). For example, IOA reduced static $\dot{q}_{HA}$ by a factor of 0.14 but reduced HA retention only 0.57-fold.

Discussion

This study is the first to demonstrate a novel, fundamental property of synovial joints, namely the existence of a coupling between joint usage and the secretion of the key arthro-protective agent HA. Intermittent cyclic joint movement almost doubled the rate of HA secretion, a response > 3 times greater than that to static joint distension (Coleman *et al.* 1997). Control studies showed that the movement-stimulated HA secretion was not attributable to inflammation or the intra-articular cannula. The significance of the key finding is discussed first, followed by an assessment of the methodology and implications at the cellular level *in situ*.

Physiological significance

Synovial fluid HA subserves tissue lubrication under low-load, high-velocity conditions to prevent inflammation and wear, and conserves synovial fluid by outflow-buffering. Movement–secretion coupling therefore enables diarthrodial joints to protect themselves against the deleterious effects of repetitive joint use. Movement-stimulated HA secretion will replace the HA lost during joint usage or prolonged immobile periods such as overnight (homeostatic role) and may even boost the fluid viscosity in joints subjected to intensive use. The secretion rates reported here are well adapted to replace the normal HA losses from the knees of conscious, caged rabbits, namely 17.6–23.1 μg h^{-1} from an endogenous HA mass of 325 μg (5.4–7.1% h^{-1} for ∼6000 kDa and 900 kDa HA, respectively; Brown *et al.* 1991). The movement–secretion coupling of HA may be part of a wider arthro-protective response to movement, since movement also stimulates chondrocyte synthesis of the lubricating glycoprotein lubricin (Nugent-Derfus *et al.* 2007).

Clinical relevance

Movement–secretion coupling provides a mechanism by which the overnight losses associated with arthritic morning stiffness can be made good, and may supply a scientific rationale for exercise therapy. Arthritic patients experience early morning stiffness and an augmented rise in serum HA on commencing daily activities, followed by a gradual relief of the stiffness (Engström-Laurent & Hällgren, 1987; Criscione *et al.* 2005). These changes are thought to reflect the clearance of periarticular oedema and escaped HA. Movement-stimulated HA secretion provides a mechanism to replace the escaped HA.

Exercise therapy can improve moderate OA (van Baar *et al.* 1999) and raise the HA-dominated synovial fluid viscosity (Miyaguchi *et al.* 2003), while immobilization reduces the HA concentration (Pitsillides *et al.* 1999). Moreover intra-articular HA injections can benefit moderate OA (Day *et al.* 2004). Movement–secretion coupling thus offers a scientific rationale for exercise therapy. In keeping with this view, the pharmacological stimulation of HA secretion improves experimental OA (Hamanishi *et al.* 1996). A better understanding of the movement–secretion coupling pathways may therefore open up new therapeutic avenues.

Mechanism of movement-stimulated secretion

A fall in HA concentration can stimulate HA secretion *in vitro* (Phillipson *et al.* 1985; Nagata *et al.* 1992; Dowthwaite *et al.* 2003) and HA washout stimulates its production in skin (Reed *et al.* 1994), lungs (Townsley *et al.* 1994), intestine (Østgaard & Reed, 1994) and synovium (Price *et al.* 1996*b*). Moved joints, however, underwent the same washout as static joints, so the extra secretion associated with movement may be related to strain/shear stress rather reduced HA concentration. Strain stimulates rabbit synoviocyte secretion *in vitro* (Momberger *et al.* 2005) and is a better stimulant than shear stress in chick embryo fibrocartilage cells (Dowthwaite *et al.* 2003). Moreover cyclic strain up-regulates HAS mRNA expression and HA secretion in chondrocytes (Yamazaki *et al.* 2003), cervical fibroblasts (Takemura *et al.* 2005) and lung fibroblasts (Mascarenhas *et al.* 2004).

A relatively short duration of strain evokes prolonged stimulation *in vitro* (Dowthwaite *et al.* 2003; Momberger *et al.* 2005). *In vivo*, however, 'static' joints were moved during the initial washouts (and briefly every 30 min for 5 h in vehicle control studies), yet had lower secretion rates than continually moved joints. This indicates that the response *in vivo* may be more short-lived (more rapid 'off' phase) than *in vitro*, or has a higher threshold for stimulus duration. Issues requiring further investigation thus include the stimulus–response curve, the nature of the synoviocyte mechanosensor, the signal pathways that couple sensor to effector, and the mechanism(s) by which the membrane HAS activity is increased.

Methodology and error assessment

The washout analysis quantified for the first time the poor miscibility of the synovial fluid HA *in vivo* and verified the need for mixing cycles (Fig. 1*B* and Supplemental material). The low k_{misc} is attributed to the high viscosity and to the complex, poorly interconnected anatomy of the cavity (Knight & Levick, 1982). Residual endogenous HA (1.9 μg for a mass of $\sim$325 μg, Appendix eqn (2)) would cause $\dot{q}_{HA}$ to be overestimated by $\leq$ 3.5%. The static $\dot{q}_{HA}$ values is not necessarily basal, due to movement during washout and a reduced negative feedback by HA concentration (see above). The effect of metabolic inhibitors confirmed that the accumulated HA is chiefly an active cellular secretion (Fig. 6), in keeping with our previous report that protein kinase C inhibition reduces the secretion rate (Anggiansah *et al.* 2003). The small effect of sodium 4-methylumbelliferone *in vivo* (24% inhibition) was similar to a 25% inhibition of pancreatic cancer cells (Morohashi *et al.* 2006) and 35% inhibition of non-stimulated keratinocytes over 6 h cf. 65% inhibition of growth factor-stimulated keratinocytes (Rilla *et al.* 2004). The marked, parallel variation in static $\dot{q}_{HA3}$, moved $\dot{q}_{HA3}$ and m_{endog} (Figs 3 and 4) may arise from variation in synovial surface area per joint, synoviocyte density and HAS activity.

The net secretion rate $\dot{q}_{HA}$ probably underestimates the true secretion rate, owing to partial trans-synovial HA escape. The secretion rates corrected for ERSHA fractional recovery, $\dot{q}_{HA}^{corrected}$ (Methods), were 21.2 μg h^{-1} in static joints, and 37.8 μg h^{-1} in moved joints. The recovery experiments probably overestimated loss, however, due to technical problems, the raised diffusion gradient and the 2.7 ml volume expansion, which increases convective escape (Sabaratnam *et al.* 2004). In keeping with this view, the 8–9% h^{-1} loss exceeded the 5.4–7.1% h^{-1} loss of 6000–900 kDa HA from rabbit knees expanded by 0.30–0.56 ml (Brown *et al.* 1991).

HA secretion *versus* clearance; turnover and homeostasis

Given a static secretion rate of 10.9–21.2 μg h^{-1}, the endogenous 325 μg HA has a turnover time of 29.8–15.3 h (rate constant k 0.034–0.066 h^{-1}; half-life $t_{1/2}$ 20.4–10.6 h), falling to 14.8–8.6 h in moved joints. Similar estimates have been derived from clearance studies, namely 14.1–18.5 h (knees, conscious rabbits; Brown *et al.* 1991), 23 h (tritiated HA, rabbit knees; Lindenhayn *et al.* 1997) and 23–40 h (sheep hock; Fraser *et al.* 1993). HA loss can also be predicted using a reflection–concentration

Table 2. Rate of hyaluronan secretion by synoviocytes compared with other cell phenotypes (picograms per cell per hour)

Cell type	pg h^{-1}	Conditions	Reference
Rabbit synoviocyte* *in situ*	2.4–4.7§	Intact knee joint, static	Present study
Rabbit synoviocyte* *in situ*	4.8–8.3§	Intact knee joint, cycled	Present study
Rabbit synoviocyte* *in vitro*	0.30	Subconfluent, serum-free	Momberger *et al.* (2005)
Rabbit synoviocyte* *in vitro*	0.47	Subconfluent, static stretch	Momberger *et al.* (2005)
Human arthritis, synovium	0.10	Serum free, *in vitro*	Recklies *et al.* (2001)
Human arthritis, synovium	0.83	IL-1β stimulated	Recklies *et al.* (2001)
Human arthritis, synovium	7.29	IL-1β + TGF-β1	Recklies *et al.* (2001)
Human synovial fibroblast*	0.083	Basal, *in vitro*	Haubeck *et al.* (1995)
Human synovial fibroblast*	1.250	TGFβ stimulated	Haubeck *et al.* (1995)
Human synovial fibroblast*	1.150	Basal *in vitro*	Yaron *et al.* (1978)
Human synovial fibroblast*	2.290	PGE_2 stimulated	Yaron *et al.* (1978)
Swiss 3T3 fibroblast	0.210	Confluent, serum-free	Kitchen & Cysyk (1995)
Swiss 3T3 fibroblast	0.960	Confluent serum-stimulated	Kitchen & Cysyk (1995)
Swiss 3T3 fibroblast	1.060	Log-growth phase	Kitchen & Cysyk (1995)
Human knee chondrocyte	0.031	Culture, serum stimulated	Recklies *et al.* (2001)
Rat keratinocyte	0.017	Basal, *in vitro*	Rilla *et al.* (2004)
Human breast CA MCF-7	0.008	High cell density, *in vitro*	Kultti *et al.* (2005)
Human breast CA MCF-7	0.810	HAS3 transfected	Kultti *et al.* (2005)
Human osteosarcoma MG-63	0.031	Culture + growth factors	Recklies *et al.* (2001)
HUVEC**	2.800	Culture, static	Gouverneur *et al.* (2006)
HUVEC	5.500	Culture, shear stress	Gouverneur *et al.* (2006)
Renomedullary interstitial cell	0.016	Subconfluent, isotonic	Hansell *et al.* (1999)
Renomedullary interstitial cell	0.001	Confluent, isotonic	Hansell *et al.* (1999)
Renomedullary interstitial cell	0.003	Subconfl. hyperosmolar	Hansell *et al.* (1999)

* 'Synoviocyte' refers here specifically to fibroblast-like synovial cell grown from microdissected intima ($\leq$ 20 μm thick). Cells grown from a mixture of synovium and the considerably thicker subsynovium or from rheumatoid tissue are designated 'synovial fibroblasts', since their anatomical origin is less well defined. ** Human umbilical endothelial cell line. § Lower value is based on observed net secretion rate, upper value is maximum estimate after correction by ERSHA fractional loss.

polarization model (Coleman *et al.* 1999). Given 3.6 mg HA per millilitre of fluid (Price *et al.* 1996*a*) and a net fluid turnover of $\sim$0.7 μl min^{-1} during normal activity (Levick, 1995), the predicted HA loss is 21.8 μg h^{-1}, in keeping with the loss of 17.6–23.1 μg h^{-1} indicated by the removal rate constants of Brown *et al.* (1991). Since the HA secretion rates (static 10.9–21.2 μg h^{-1}, moved 22.0–37.8 μg h^{-1}) are broadly similar to the normal trans-synovial escape rates they are well adapted to maintain intra-articular HA homeostasis.

Synoviocytes are specialized HA secretors

Synovium is thought to be the source of the HA because it has a greater surface area, cell density and HAS activity (Table 2) than cartilage, where HA is bound in a supra-molecular complex. Approximately 4.6 million synoviocytes abut the rabbit knee synovial cavity (area 11.83 cm^2, Knight & Levick, 1983; cell fraction 0.69, synoviocytes 67%, mean cell width 12.3 μm, Levick & McDonald, 1989). The secreted HA must derive mainly from these surface cells because cellularity declines sharply with depth (Price *et al.* 1995) and the matrix permeability to HA is low (Sabaratnam *et al.* 2004). Based on these considerations the synoviocytes *in situ* secrete 2.4–4.7 pg h^{-1} per cell (static) to 4.8–8.3 pg h^{-1} (moved). This is $\sim$10 times faster than synoviocytes *in vitro* (Table 2), indicating that the phenotype is greatly influenced by its microenvironment; and it is 10–100 times faster than many other cell types (Table 2). Appropriately, synoviocytes express high levels of UDP-glucose dehydrogenase (Pitsillides *et al.* 1993; Pitsillides, 2003). They also show a uniquely massive secretory response to IL-1β plus TGF-β1 (Table 2). Synoviocytes *in situ* must thus be regarded as prolific, highly specialized, regulated HA secretors.

HA synthesis is a substantial metabolic burden

To generate the observed static $\dot{q}_{HA}$, 1.9×10^{12} chains of 3500 kDa HA are synthesized per hour, incorporating 3.4×10^{16} glucose residues per hour (dimer

mol.mass 379 Da). Total synovial glucose consumption is $\sim 1.6 \times 10^{17}$ molecules h^{-1} at rest ($\sim$2 mg glucose h^{-1} $(cm^3$ tissue$)^{-1}$; synovial area 11.83 cm^2, thickness $\sim 20 \times 10^{-4}$ cm; Levick, 1995). The HA synthesis accounts for $\sim$23% of the resting glucose consumption and is thus a substantial metabolic burden. Demand is readily met, however, by the synovial plasma flow of 45–90 μl min^{-1}, which delivers $(8–16) \times 10^{18}$ glucose molecules h^{-1}.

HAS density in synoviocytes

Since the HA chain is synthesized at a rate of $\sim$60–200 disaccharides min^{-1} (Kitchen & Cysyk, 1995; Asplund *et al.* 1998; Bodevin-Authelet *et al.* 2005), it takes 46–154 min to synthesize one 3500 kDa chain of 9235 disaccharides. A static synoviocyte secreting 6882 chains min^{-1} (2.4 pg h^{-1}) therefore requires 316 559–1 060 400 HAS molecules. This implies a very dense packing of HAS in the synoviocyte membrane and a high concentration of HA chains sprouting from the cell surface, again highlighting the specialized phenotype of synoviocytes *in situ*.

Conclusion

The present study has demonstrated a novel coupling between joint movement and synovial HA secretion. The coupling serves to protect joints during prolonged use and to replace HA lost during periods of immobility, such as overnight. Movement–secretion coupling also offer a potential link between clinical benefit and exercise therapy in moderate osteoarthritis.

Appendix

Washout curve analysis; equivalent residual volume, HA miscibility and estimate of recovery residue

A surprisingly large number of washes was needed for maximal HA recovery, prompting a formal analysis of the washout process. If intra-articular HA were perfectly miscible after the mixing cycles, it would distribute uniformly in the introduced wash. Under these conditions the residual intra-articular solute mass m_{ia} after n washes of volume V_w obeys the power relation

$$m_{ia(n)} = m_{endog}[V_r/(V_w + V_r)]^n \quad (1)$$

where m_{endog} is the starting mass, V_w is wash volume and V_r is the residual volume after aspirating each wash. By contrast, if the highly viscous HA fails to mix completely with the wash, the relation becomes

$$m_{ia(n)} = m_{endog}[V_e/(V_w + V_e)]^n \quad (2)$$

where V_e is an equivalent or virtual residual volume greater than V_r. Washout is now slower than predicted by eqn (1), as it was in practice (Fig. 1). To interpret V_e, we defined the miscibility coefficient, k_{misc}, as the fraction of the intra-articular HA that passes into the Ringer solution during each wash. This leads to the relation

$$m_{ia(n)} = m_{endog}\{1 - [k_{misc}V_w/(V_w + V_r)]\}^n. \quad (3)$$

From eqns (2) and (3), $V_e = [(V_w + V_r)/k_{misc}] - V_w$. Equations (2) and (3) predict a linear relation between the known parameters n and the logarithm of $m_{ia(n)}$. The slope s of the logarithmic plot is the removal rate constant (fraction removed per wash) and equals $\ln[V_e/(V_w + V_e)]$ or equivalently $\ln\{1 - [k_{misc}V_w/(V_w + V_r)]\}$. The washout rate constant s was therefore determined by linear regression analysis and used to evaluate V_e and k_{misc}:

$$V_e = V_w e^s/(1 - e^s) \quad (4a)$$

$$k_{misc} = [(V_w + V_r)/V_w](1 - e^s). \quad (4b)$$

The practical value of the washout analysis was twofold. First, it provided a quantitative measure, k_{misc}, for assessing the effect of the washout variables (number of mixing cycles, wash volume V_w, wash temperature; see Supplemental material). Second, the residual intra-articular HA mass after 18 washes can be calculated from eqns (3) and (4) to estimate the residual HA error (see Discussion).

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Supplemental material

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